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Virus infection causes specific learning deficits in honeybee foragers

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In both mammals and invertebrates, virus infections can impair a broad spectrum of physiological functions including learning and memory formation. In contrast to the knowledge on the conserved mechanisms underlying learning, the effects of virus infection on different aspects of learning are barely known. We use the honeybee (*Apis mellifera*), a well-established model system for studying learning, to investigate the impact of deformed wing virus (DWV) on learning. Injection of DWV into the haemolymph of forager leads to a RT-PCR detectable DWV signal after 3 days. The detailed behavioural analysis of DWV-infected honeybees shows an increased responsiveness to water and low sucrose concentrations, an impaired associative learning and memory formation, but intact non-associative learning like sensitization and habituation. This contradicts all present studies in non-infected bees, where increased sucrose responsiveness is linked to improved associative learning and to changes in non-associative learning. Thus, DWV seems to interfere with molecular mechanism of learning by yet unknown processes that may include viral effects on the immune system and on gene expression.

Keywords: *Apis mellifera*; virus; learning; memory; responsiveness

1. INTRODUCTION

In vertebrates, viral infections can cause defects in the morphology and in the function of the nervous system including a wide range of impairments in cognitive and motor function, but also social behaviour (Tomonaga 2004; Beraki *et al.* 2005). In human brains, HIV infection selectively damages the cortex and affects brain regions that control language, sensory and motor functions (Thompson *et al.* 2005). Viral infection in mice causes neurobiological impairments and alterations in aggressive behaviour, cognitive ability, locomotor activity and deficits in spatial reference memory (Kamitani *et al.* 2003; Beraki *et al.* 2005). Even offspring of mice infected by the influenza virus show deficiencies in exploratory behaviour and social interaction (Shi *et al.* 2003). Observations from a variety of insect species also reveal evidence for the effects of viral infection on developmental processes, locomotor activity, feeding, mating and other behaviours (Platt *et al.* 1997; Burand *et al.* 2005; Kamita *et al.* 2005; Vasconcelos *et al.* 2005).

This—together with the high conservation of molecular processes underlying learning (Kandel 2001)—prompted us to study the impact of viral infection on different, well-characterized forms of learning in insects. The honeybee (*Apis mellifera*) provides an ideal model organism in this respect, since honeybees show a rich and well-investigated behavioural repertoire (Menzel & Müller 1996) and can be infected by more than 18 different viruses (Bailey & Ball 1991; Allen & Ball 1996).

Here, we study the impact of controlled infection with deformed wing virus (DWV) on sensory perception, non-associative and associative learning in adult honeybees. DWV infections are very abundant and can be detected in up to 90% of colonies (Tentcheva *et al.* 2004; Berenyi *et al.* 2006). While DWV infection in early development leads to deformation of wings, paralysis and mortality of the emerging bees (Lanzi *et al.* 2006), the infection of adult bees does not show such deformation. However, the DWV-infected colonies suffer from weakness, depopulation and sudden collapse (Berenyi *et al.* 2006).

In honeybees, the proboscis extension response (PER), which is elicited by an appetitive stimulus to antennae or proboscis, allows for testing sensory capabilities, non-associative as well as associative forms of learning (Menzel & Müller 1996). Our results show that controlled infection of honeybee foragers by DWV in the laboratory causes specific impairments in sensory responsiveness and associative learning, while non-associative learning remains intact.

2. MATERIAL AND METHODS

(a) *Collection and screening of virus-infected honeybees*

Adult bees were collected from hives owned by beekeepers near Saarbrücken, Germany (winter to spring 2006). At the collection site, bees were directly frozen and stored in liquid nitrogen until RNA extraction and analysis.

(b) *RNA extraction*

Five adult bees from each colony were pooled and homogenized in liquid nitrogen and 80–100 mg of the homogenized tissues was mixed with 1 ml Trizol reagent (Invitrogen, Germany) according to the manufacturer's instructions. To reduce the level of contamination with proteoglycans and polysaccharides, RNA was precipitated from the aqueous phase using 0.25 volume of isopropanol and 0.25 volume of buffer containing 1.2 M NaCl and 0.8 M sodium citrate (Chomczynski & Mackey 1995). After centrifugation, RNA pellets were resuspended in DEPC-treated water containing 0.1 mM

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EDTA (pH 6) and quantified by a spectrophotometer. RNA samples were stored at −80°C in the presence of ribonuclease inhibitor (Fermentas, Germany) for subsequent use.

(c) *Oligonucleotide primers*

For detecting DWV infection in the honeybees, DWV-specific primers (CCTGCTAATCAACAAGGACCTGG) and (CAGAACCAATGTCTAACGCTAACCC) that lead to a fragment of 355 bp were used according to a previous work by Genersch (2005). Actin was used as a control and the specific primers were designed from available honeybee sequences (NC_007085) (National Center for Biotechnology Information, NCBI). The actin-specific primers, (CAGGACGCA CTACAGGCAT) and (CACGCTCTGCGGTAGTGGT), lead to a fragment with an expected length of 738 bp.

(d) *Reverse transcription and PCR amplification*

A two-step reverse transcription polymerase chain reaction (RT-PCR) protocol was used for the diagnosis of DWV from extracted RNA. Reverse transcription was carried out with an average of 1 µg RNA, random hexamer primer and MoMuLV reverse transcriptase (Fermentas, Germany) according to the manufacturer's protocol. PCR amplification was performed with 5 µl cDNA, appropriate specific primers, Taq polymerase and 2 mM $MgCl_2$ (Fermentas, Germany) in 20 µl total reaction mixture. The mixture was heated at 95°C for 10 min, followed by 35 amplification cycles under following conditions: 95°C for 30 s, 58°C for 1 min and 72°C for 1 min followed by 72°C for 10 min to complete the polymerization. A negative control containing water instead of RNA and a DWV-positive control (provided by Reinhold Siede, Bieneninstitut Kirchhain, Germany) was included in each RT-PCR experiment. PCR products were analysed by 1% agarose gel electrophoresis.

(e) *Animal collection for behaviour and controlled infection with DWV*

Adult honeybees were caught from a hive that had been tested negative for viral infections. After immobilization on ice, the bees were mounted in small metal tubes. The animals were fed (1 M sucrose) each evening to satiation and kept in darkness at a relative humidity of 70% and at 20–25°C until used for controlled infection and behavioural tests (Müller & Hildebrandt 2002). For controlled infection, the DWV lysate and control lysate was extracted from DWV-infected bees or non-infected bees, as verified by RT-PCR. Five bees from each group were homogenized in liquid nitrogen and mixed with 5 ml phosphate-buffered saline (PBS, pH 7.4). The samples were centrifuged at 3000 r.p.m. for 30 min at 4°C and the supernatant was stored in aliquots at −20°C for future use. The DWV lysate and control lysate were diluted 1 : 1000 in PBS and 2–4 µl was injected into the haemolymph of each bee using a microcapillary (Müller & Hildebrandt 2002). For oral application of DWV, honeybees were kept in small cages provided with sucrose paste containing either DWV lysate or control lysate at a dilution of 1 : 100. RT-PCR was used to monitor the level of DWV infection at different days after haemolymph injection and at the seventh day after oral application.

(f) *Responsiveness to appetitive stimuli*

Responsiveness of the bees to appetitive stimuli was tested by using the proboscis extension response (PER). An antenna was stimulated by a series (inter-trial interval, 2 min) of defined stimuli with gradually increasing sucrose concentrations (0 M, 30 mM, 100 mM, 300 mM and 1 M). For each sucrose stimulus, the PER was monitored and used as measurement of sucrose responsiveness for each bee (Friedrich *et al*. 2004; Scheiner 2004).

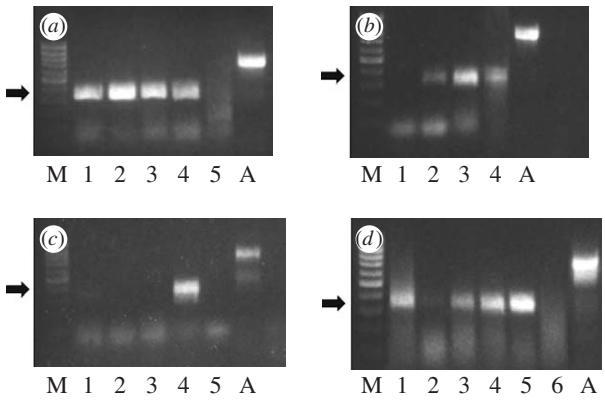

Figure 1. Qualitative diagnosis of DWV using RT-PCR. In all gels, the arrow indicates the DWV product; M, marker; A, actin control. (*a*) DWV infection: lane 1–4, RT-PCR from different infected samples; lane 5, negative control (water). (*b*) Localization of DWV in body regions of honeybee: lane 1, head; lane 2, thorax; lane 3, abdomen; lane 4, DWV-positive control. (*c*) Oral application of DWV: lane 1, seventh day after continuous oral application of DWV lysate in sucrose; lane 2, seventh day after continuous oral application of control lysate in sucrose; lane 3, seventh day after continuous oral application of sucrose; lane 4, DWV-positive control; lane 5, negative control (water). (*d*) Time course after DWV haemolymph injection: lane 1, DWV-positive control; lane 2–5 represent second to fifth day after DWV lysate injection, respectively; lane 6, negative control (water).

(g) *Non-associative learning tests*

(i) *Sensitization*

Two minutes after testing the initial responsiveness to an odour stimulus, the honeybee was sensitized by antennal stimulation with sucrose (1 M). After 20 s, the second odour stimulus was presented to test for sensitization.

(ii) *Habituation*

Habituation of PER was tested by repeated stimulation of an antennae (1 M sucrose) at an inter-stimulus interval of 1 s. The number of PER occurring before five consecutive response failures defines the habituation criterion (Müller & Hildebrandt 2002).

(h) *Associative olfactory learning*

Associative conditioning was performed as described earlier (Müller 2002). A conditioning trial comprises pairing an odour stimulus (carnation) (conditioned stimulus, CS) with a sucrose reward (1 M) (unconditioned stimulus, US). After the animals received three successive conditioning trials at an inter-trial interval of 2 min, memory tests were performed 2 and 24 h after training.

3. RESULTS

(a) *Detection and characterization of DWV infection*

The RT-PCR-based screening revealed strong DWV signals in bees collected from colonies that died off during the winter (figure 1*a*). In these bees, we found no evidence for infections by other viruses (acute bee paralysis virus,

sacbrood bee virus, Kashmir bee virus and black queen cell virus) as tested by RT-PCR. Despite the strong DWV infection, there were no symptoms for wing deformation, emphasizing the importance of PCR-based molecular diagnosis to verify viral infections in honeybees. Repeated RT-PCR experiments show that the DWV signal is high in the abdomen and gradually decreases in the thorax and head (figure 1*b*).

To test for a possible transfer of DWV via food, groups of bees were fed with sucrose alone or sucrose contaminated with control or DWV lysate. DWV infection in the animals was tested at different times after infection using RT-PCR. As shown in figure 1*c*, these feeding experiments did not lead to a detectable infection by DWV within the tested time window of 7 days. Thus, oral application does not lead to a DWV infection at all, or it takes much longer to reach the threshold level for DWV infection.

Injection of DWV lysate directly into the haemolymph of bees causes a strong RT-PCR signal when compared with the control groups injected with control lysate or PBS. A signal is visible 3 days after injection and the signal increases gradually from 3 to 5 days (figure 1*d*). Thus, injection is suited for a controlled DWV infection of bees for the behavioural experiments. Although there is no difference in the survival rate between both groups, the DWV lysate-injected bees show a slight decrease in motor activity (e.g. slow extension of the proboscis) after sucrose stimulation.

(b) *DWV infection affects sucrose responsiveness*

Honeybees provide the opportunity to study non-associative learning—such as sensitization and habituation—as well as associative olfactory learning with single animals. Since these learning paradigms are based on the proboscis extension response (PER) elicited by appetitive stimuli like sucrose, it is necessary to test whether processing of this appetitive stimuli is affected by DWV infection.

Based on the results derived from RT-PCR measurement, we tested the responsiveness of bees at two time points during DWV infection: the first and the fourth day after injection (figure 1*d*). At these time points, we verified the level of DWV infection with RT-PCR and tested whether an antennal stimulation with sucrose elicits the PER. On the first day after injection, the responsiveness of DWV-infected bees does not differ from that of the control group (figure 2*a*). However, on the fourth day after injection, the responsiveness of the DWV-infected group to water and low sucrose concentration is strongly increased when compared with the control group (figure 2*b*). Since there are no differences in the responsiveness to high sucrose concentrations between DWV-infected and control bees, we use 1 M sucrose as appetitive stimuli in the following experiments.

(c) *Sensitization and habituation of the PER*

Sensitization is the increased responsiveness to a neutral sensory stimulus (odour) shortly after application of a stimulus (sucrose) that arouses the animal. DWV-infected bees do not differ from control bees. In both groups, the first odour stimulus elicits PER only in a few bees (less than 10%), while the arousing stimuli (1 M sucrose) leads to high levels of PER (more than 90%). Odour stimulation immediately after arousal also triggers PER in only a few animals (less than 20%).

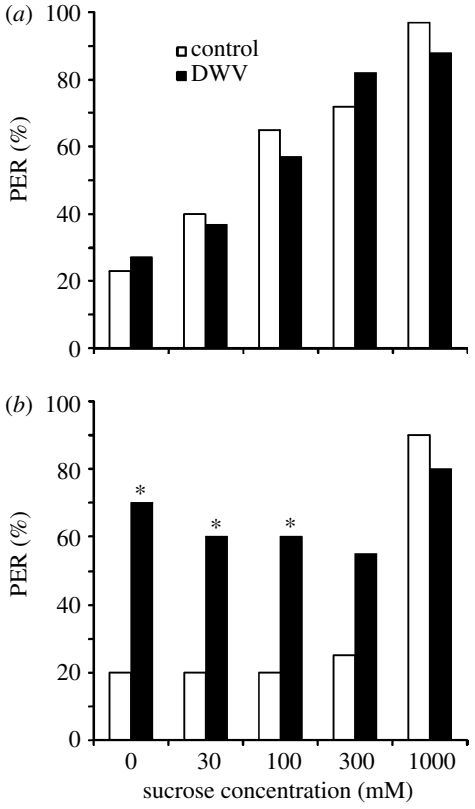

Figure 2. Sucrose responsiveness of bees was tested on the (*a*) first and (*b*) fourth day after artificial haemolymph infection with control lysate and DWV lysate. After each stimulation of an antenna with successive single stimuli of increasing sucrose concentrations (0–1 M), the PER (proboscis extension response) was recorded. The data show the mean responsiveness indicated by the percentage of PER. (First day, $n=60$ bees each group and fourth day, $n=20$ bees each group). The asterisks indicate significant difference between the groups (Fisher exact test; $^{*}p<0.05$).

Habituation represents the gradual decrease in responsiveness during a continuous series of repeated stimulations. When the bee is habituated, sucrose stimuli will no longer elicit PER. Testing habituation from the first day until the fourth day after injection did not reveal any difference between the DWV-infected group and the control group. Since DWV infection in both groups has been verified by RT-PCR, these results show that habituation is not affected by DWV.

(d) *Associative olfactory learning*

The well-established associative olfactory conditioning paradigm consists of the pairing of an odour stimulus (CS) with a sucrose reward (US). Figure 3 shows that DWV-infected bees show a significantly reduced acquisition when compared with control bees. Moreover, memory retention as tested 2 and 24 h after conditioning is also low in DWV-infected bees. Thus, DWV infection seems to have specific effects on neuronal signalling processes because DWV infection only impairs associative learning without affecting non-associative processes.

4. DISCUSSION

In our behavioural analysis, we demonstrate that DWV infection of adult forager bees leads to specific impairments in sucrose responsiveness and associative olfactory

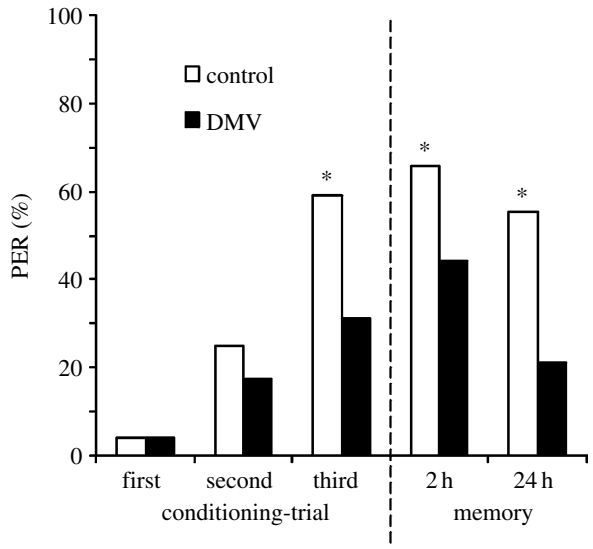

Figure 3. Olfactory conditioning of PER on the third day after infection with control lysate and DWV lysate. The animals were conditioned by pairing an odour stimulus (CS) with a subsequent sucrose reward (US) to the antenna and proboscis. Memory retention was tested on the fourth day after infection by stimulation with odour alone. The data show the percentage of PER elicited after CS stimulation in the control lysate-injected ($n=49$) and DWV lysate-injected ($n=50$) group. The asterisks indicate significant differences between the groups (χ^2 test; $^*p<0.05$).

learning but does not affect non-associative forms of learning like sensitization and habituation. Only direct haemolymph infection by DWV is effective and leads to a strong replication of the virus, while oral application is ineffective, which is in agreement with other studies (Bailey & Ball 1991). However, since we tested only up to 7 days of continuous oral application, we cannot exclude that a long-lasting oral uptake of DWV-contaminated food during hibernation may be a source of DWV infection. Thus, under natural conditions, haemolymph infection via parasites like *Varroa destructor* as proposed by Bailey & Ball (1991) is the most probable scenario for DWV transmission. Although the transmitted material (virus, bacteria, etc.) has not been specified, a recent report demonstrates that foragers infested by *Varroa destructor* are affected in non-associative forms of learning (Kralj *et al.* 2007).

In agreement with results on queens and drones (Chen *et al.* 2006), our RT-PCR measurements of DWV-infected foragers showed a maximal signal in the abdomen, followed by the thorax and head of the adult honeybee. Although viral infection in the head is quite weak, it may interfere with molecular mechanisms underlying learning at different levels. So, it is feasible that a viral infection interferes with signalling cascades underlying learning by triggering the immune system. This is supported by the observation that injection of lipopolysaccharide (LPS), which triggers immune response, affects olfactory conditioning but not sensitization in honeybees (Mallon *et al.* 2003; Riddell & Mallon 2006). This impairment in associative learning is observed approximately 3 days after LPS injection, which is in agreement with our study. Interestingly, LPS injection and viral infections also cause learning deficits in mammals (Weed & Gold 2001; Sparkman *et al.* 2005). Our study however shows that, based on the present knowledge, a simple explanation of the potential molecular targets affected by DWV is yet not possible.

The unique combination of enhanced sucrose responsiveness, normal non-associative learning and defects in associative learning caused by DWV infection has not yet been observed. All previous reports show that elevated sucrose responsiveness is always combined with an improved associative learning performance, which is in clear contrast to our observations. The genotype, the role of the bees in foraging, the satiation level and many other parameters affect sucrose responsiveness and—in parallel—very defined features of associative learning (Scheiner *et al.* 2001; Friedrich *et al.* 2004; Scheiner 2004). These strongly linked physiological processes depend to a great extent on molecular processes mediated by the biogenic amine octopamine. While injection of octopamine elevates sucrose responsiveness and enhances acquisition, inhibition of octopaminergic transmission decreases responsiveness and impairs acquisition (Hammer & Menzel 1998; Menzel *et al.* 1999; Scheiner *et al.* 2002). All this is in contrast to our demonstrated effects of DWV infection on sucrose responsiveness and acquisition and points to yet unknown processes affected by DWV. Our finding that DWV infection does not interfere with habituation supports this notion. Processes that affect sucrose responsiveness and associative learning also affect habituation (Braun & Bicker 1992; Müller & Hildebrandt 2002; Scheiner 2004). Thus, the discrepancy between the current knowledge about the interaction between sucrose responsiveness, non-associative and associative learning in bees and the behavioural effects after DWV infection demands a search for new explanations. Since the effects on behaviour are observed days after infection, it is feasible that viral infections (possibly also LPS injections) lead to changes in the gene expression pattern, which has been reported in mice (Linnea *et al.* 2005). So, future studies have to show whether gene expression patterns that change during development and caste differentiation of bees (Whitfield *et al.* 2003) are targets of viral infections.

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